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Altered Glycosylation of Membrane Glycoproteins Associated with Human Mammary Carcinoma

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N-Linked sugar chains of normal mammary gland, mammary carcinomas (primary lesion), and axillary lymph node metastases of mammary carcinomas were released from their membrane preparations by hydrazinolysis and their structures were analyzed. Fractionation using a *Datura stramonium* agglutinin (DSA)-Sepharose column revealed that the metastasized carcinomas contain more than twice as much DSA-binding oligosaccharides as the normal gland, and the primary carcinomas contain an intermediate amount. These oligosaccharides were elucidated to have tri- and tetraantennary structures containing the GlcNAcβ1→6(GlcNAcβ1→2)Man group with and without *N*-acetyllactosamine repeating units. Lectin blot analysis of membrane glycoproteins and histochemical staining of tissues using biotinylated DSA indicated that these glycosylation changes predominantly occur in a limited number of glycoproteins with apparent molecular weights of 90, 160, and 210 kilodaltons, and mammary carcinomas are distinguishable from normal gland by their intense intracytoplasmic staining.

Key words: Human mammary carcinoma — Metastasis — *N*-Linked sugar chain

It has been shown that the altered glycosylation of membrane glycoproteins caused by oncoviral transformation of rodent cells involves an increase of the GlcNAcβ1→6 linkage attached to the Manα1→6 arm of the trimannosyl cores of complex-type sugar chains.[1,2] This phenotypic change is supposed to be under the control of some specific regions of oncoviral genomes and closely associated with tumorigenic potential of the transformed cells.[3] Recent studies on experimental metastasis of murine tumor cells have suggested that this change in sugar moieties correlates with the expression of metastatic phenotypes of tumor cells.[4,5]

In human carcinomas, however, information about the altered glycosylation of cellular glycoproteins is still very limited, although it has been reported that glycoproteins obtained from human malignant breast lesions show high reactivity to leukoagglutinating phytohemaglutinin (L-PHA[5]), which is used as a probe for detection of the GlcNAcβ1→6Manα1→branching,[6] while those from normal tissues and benign lesions show low reactivity.[7] On the basis of chemical analysis of oligosaccharide structures, we have also shown that increased expression of this branching occurs in human esophageal squamous carcinoma.[8] These lines of evidence, though fragmentary, suggest that the altered branch formation of *N*-linked sugar chains widely observed in the rodent system may also exist in human cancers. To confirm this, analysis of a variety of human cancer cells is indispensable. From the viewpoint of clinical value, it is particularly important to know whether this glycosylation change is associated with metastasis.

In this study, *N*-linked sugar chains of cellular glycoproteins from human mammary carcinomas, which had metastasized to the axillary lymph node, were compared structurally and histochemically with those from the primary lesion and from normal mammary tissues.

MATERIALS AND METHODS

Chemicals, lectins, and enzymes NaB^3H_4 (360 mCi/mmol) and NaB^2H_4 were purchased from New England Nuclear, Boston, MA and Merck Co., Darmstadt, respectively. Concanavalin A (Con A)-Sepharose was purchased from Pharmacia, Ltd., Uppsala and *Datura stramonium* agglutinin (DSA)-Sepharose was prepared by the method of Yamashita *et al.*[9] Glycosidases used in this study were prepared and used as described previously.[3]

Oligosaccharides Mono-, di- and trisialyl derivatives of Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4Glc-NAc_{OT} ($Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$), and mono- and disialyl derivatives

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[5] The abbreviations used are: L-PHA, leukoagglutinating phytohemagglutinin; Con A, concanavalin A; DSA, *Datura stramonium* agglutinin; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose.

of Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ ($Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$), in which subscript OT indicates NaB^3H_4-reduced oligosaccharides, were prepared from fetuin by hydrazinolysis.[10] Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ ($Gal_4 \cdot GlcNAc_4 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$), Gal$\beta$1→4GlcNAc$\beta$1→2Man$\alpha$1→6[Gal$\beta$1→4GlcNAc$\beta$1→4(Gal$\beta$1→4GlcNAc$\beta$1→2)Man$\alpha$1→3]Man$\beta$1→4GlcNAc$\beta$1→4(Fuc$\alpha$1→6)GlcNAc$_{OT}$ (2,4-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$), Gal$\beta$1→4GlcNAc$\beta$1→6(Gal$\beta$1→4GlcNAc$\beta$1→2)Man$\alpha$1→6(Gal$\beta$1→4GlcNAc$\beta$1→2Man$\alpha$1→3)Man$\beta$1→4GlcNAc$\beta$1→4(Fuc$\alpha$1→6)GlcNAc$_{OT}$ (2,6-branched $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$), Gal$\beta$1→4GlcNAc$\beta$1→2Man$\alpha$1→6(Gal$\beta$1→4GlcNAc$\beta$1→2Man$\alpha$1→3)Man$\beta$1→4GlcNAc$\beta$1→4(Fuc$\alpha$1→6)GlcNAc$_{OT}$ ($Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot Fuc \cdot GlcNAc_{OT}$) and their non-fucosylated forms were obtained from recombinant erythropoietin.[11] Other standard oligosaccharides were prepared by exoglycosidase digestion of the oligosaccharides listed above.

Analytical methods High-voltage paper electrophoresis was carried out at 80 V/cm for 2 h by using pyridine-acetate buffer, pH 5.4. Bio-Gel P-4 column chromatography was carried out as described previously.[12] Methylation analysis was performed[13] using a DB5-30N column (0.25 mm × 30 m) (J & W Scientific, Cordova, CA) and a JEOL DX-300 gas chromatograph-mass spectrometer (JEOL, Tokyo). Lectin column chromatography using Con A-Sepharose and DSA-Sepharose was performed as previously described.[8,9]

Isolation of normal mammary gland and mammary carcinomas From a patient having mammary carcinoma with axillary lymph node metastasis (M.A., 50 years old, blood group B), specimens of normal mammary gland, the primary lesion, and the axillary lymph node metastasis were obtained by surgery, and designated NG-1, MC-1, and MC-1N, respectively. From another patient (C.K., 38 years old, blood group B), specimens of the primary lesion and axillary lymph node metastasis were also obtained, and designated MC-2 and MC-2N, respectively.

Preparation of membrane glycoprotein mixtures Tissues were chopped finely with scissors and suspended in ice-cold 5 m*M* Tris-buffered saline (pH 9.0) for 30 min to be disrupted. The cell suspension was then subjected to the centrifugation procedure for preparation of the membrane fraction as previously described.[1]

Release of *N*-linked sugar chains from membrane glycoproteins The dry membrane glycoprotein mixtures (120 mg) were subjected to hydrazinolysis[14] to release *N*-linked sugar chains. The oligosaccharide mixtures were purified as described previously.[1] One-fourth of them was reduced with NaB^3H_4 and the remainder was reduced with NaB^2H_4 for methylation analysis. The yields of radioactive oligosaccharides were 4.5–5.0 × 10^6 cpm for each sample.

Lectin staining of membrane glycoproteins Carcinoma tissues (wet 0.5 g) were homogenized in a Polytron homogenizer in 3 ml of 50 m*M* phosphate buffer, pH 7.4, containing 50 m*M* EDTA, 1% *n*-octylglucoside, 0.5% SDS and 1 m*M* phenylmethylsulfonyl fluoride and stirred at 4°C for 12 h. The lysates were centrifuged at 10,000*g* for 30 min, and an equal aliquot of each supernatant was subjected to 7.5% SDS-polyacrylamide gel electrophoresis[15] and then transferred to nitrocellulose membrane. After blocking with 1% bovine serum albumin, the membrane was digested with sialidase from *Arthrobacter ureafaciens* (0.5 unit/ml in 0.05 *M* acetate buffer, pH 5) at 37°C for 1 h, and washed with phosphate-buffered saline (PBS). Then, the membrane was incubated with biotinylated DSA (2 μg/ml in PBS) and visualized with the avidin-biotinylated peroxidase complex (ABC) kit (Vector Laboratories Inc., Burlingame, CA) and diaminobenzidine substrate according to the manufacturer's instructions.

Histochemical staining of tissues by DSA All tissues used in this study were fixed in formalin, embedded in paraffin and cut into 5 μm serial sections. The sections were attached to glass slides and stained as described previously.[16] Briefly, the sections were deparaffinized, hydrated through graded alcohols, incubated with fresh 0.3% H_2O_2 in methanol for 10 min, and then washed three times with phosphate-buffered saline (PBS). After digestion with sialidase (0.5 unit/ml) from *A. ureafaciens* at 37°C for 1 h and washing with PBS, the sections were incubated with biotinylated DSA (2 μg/ml in PBS containing 1% bovine serum albumin) at room temperature for 30 min and then washed three times with PBS. The sections were incubated with avidin-peroxidase conjugate (10 μg/ml) for 30 min and washed with PBS three times. Finally, the sections were reacted with 0.1% diaminobenzidine substrate and 0.02% H_2O_2 for 5 min, rinsed with the buffer, stained with hematoxylin-eosin, washed and observed under the microscope. Negative controls were prepared by incubating the sections with the biotinylated lectin in the presence of 1% *N*-acetylglucosamine oligomers.

RESULTS

Fractionation of oligosaccharides by paper electrophoresis The radioactive oligosaccharide mixtures released from normal human mammary gland (NG-1), primary mammary carcinomas (MC-1 and MC-2), and mam-

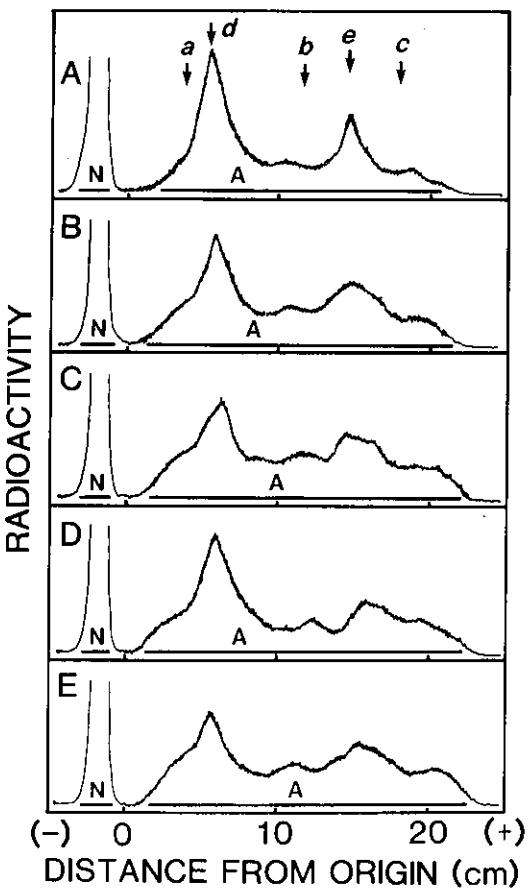

Fig. 1. Paper electrophoretograms of the radioactive oligosaccharides released by hydrazinolysis from membrane glycoproteins of normal mammary gland and mammary carcinomas. A, B, C, D and E represent the data obtained from NG-1, MC-1, MC-1N, MC-2 and MC-2N, respectively. Arrows a, b and c are positions of authentic mono-, di- and trisialylated $Gal_3 \cdot GlcNAc_3 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, and arrows d and e are those of mono- and disialylated $Gal_2 \cdot GlcNAc_2 \cdot Man_3 \cdot GlcNAc \cdot GlcNAc_{OT}$, respectively.

mary carcinomas metastasized to the regional lymph node (MC-1N and MC-2N) were subjected paper electrophoresis at pH 5.4. As shown in Fig. 1, each of the samples was separated into neutral (N) and acidic (A) peaks. Molar ratios of fractions N and A from each sample calculated on the basis of their radioactivities were as follows; NG-1, 28:72; MC-1, 27:73; MC-1N, 33:67; MC-2, 26:74; MC-2N, 31:69. The acidic fractions obtained from five samples were mostly converted to neutral oligosaccharides (AN) by sialidase digestion (data not shown) indicating that the acidic nature of the oligosaccharides in the acidic fraction A can be ascribed to sialic acid residues.

Table I. Fractionation of Oligosaccharides Obtained from Normal Mammary Gland and Mammary Carcinomas by Lectin Column Chromatography

Fractions[a]	Mammary tissues				
	NG-1	MC-1	MC-1N	MC-2	MC-2N
Neutral oligosaccharides (N)					
Con A^-	7[b]	4	9	9	11
Con A^+	12	11	7	12	6
Con A^{2+}	9	12	17	5	14
Asialo-oligosaccharides (AN)					
Con A^-	20	23	33	23	38
DSA^+	10	14	20	13	25
DSA^r	3	5	6	3	7
DSA^-	7	4	7	7	6
Con A^+	48	46	31	49	29
Con A^{2+}	4	4	3	2	2

a) Symbols are defined in the text.
b) Each value is the percent molar ratio of oligosaccharides in a fraction to the total oligosaccharides.

Fractionation of oligosaccharides by serial lectin column chromatography and Bio-Gel P-4 column chromatography Both neutral oligosaccharide fractions N and asialo-oligosaccharide fractions AN were further fractionated by lectin column chromatography. Each sample was first applied to a Con A-Sepharose column and separated into three fractions; the fraction unbound to the column (Con A^-), and the fractions bound to the column and eluted with 5 m*M* α-methyl glucopyranoside (Con A^+) and then with 100 m*M* α-methyl mannopyranoside (Con A^{2+}). As summarized in Table I, the carcinomas (MC-1N and MC-2N) metastasized to the axillary lymph node contained higher amounts of the neutral and asialo-oligosaccharides recovered in the Con A^- fractions and less of those in the Con A^+ fractions as compared with others. The metastasized carcinomas also showed the increase of the Con A^{2+} fraction of neutral oligosaccharides. It is well known that high mannose- and/or hybrid-type oligosaccharides are recovered in the Con A^{2+} fraction, biantennary complex-type oligosaccharides in the Con A^+ fraction, and highly branched complex-type oligosaccharides in the Con A^- fraction.[17] Actually, the Con A^{2+} fraction of the neutral oligosaccharide and the Con A^+ fractions were elucidated to consist of a series of high mannose-type and biantennary complex-type oligosaccharides, respectively, by analyses in the same manner as previously described[1] (data not shown).

Higher contents of the Con A^- fractions of asialo-oligosaccharides in metastasized carcinomas MC-1N and MC-2N suggest that increased branching of the complex-type sugar chains may occur concomitantly with acquisition of metastatic potential of human mammary carcino-

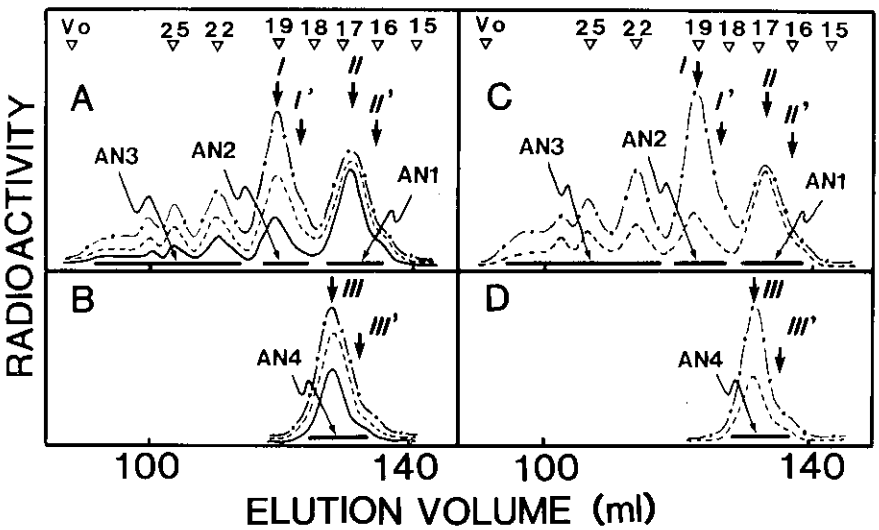

Fig. 2. Bio-Gel P-4 column chromatography of asialo-oligosaccharides obtained from normal mammary gland and mammary carcinomas. A and B, the DSA^+ and the DSA^r fractions of NG-1 (solid line), MC-1 (dotted line) and MC-1N (dot-dashed line) obtained from a patient (M.A.), respectively; C and D, the DSA^+ and the DSA^r fractions of MC-2 (dotted line) and MC-2N (dot-dashed line) obtained from another patient (C.K.), respectively. White triangles indicate elution positions of glucose oligomers (numbers indicate the glucose units) and the void volume (V_0). Arrows I to III are elution positions of authentic oligosaccharides: I, Gal_4·$GlcNAc_4$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; II, 2,6-branched Gal_3·$GlcNAc_3$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; III, 2,4-branched Gal_3·$GlcNAc_3$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. Arrows I′, II′, and III′ are those of non-fucosylated forms of I, II, and III, respectively.

mas. In order to confirm this point, the Con A^- fraction of each sample was then subjected to DSA-Sepharose column chromatography. Complex-type oligosaccharides with the GlcNAcβ1→6(GlcNAcβ1→2)Man group strongly bind to the DSA column and are eluted with 1% *N*-acetylglucosamine oligomer, while those with the GlcNAcβ1→4(GlcNAcβ1→2)Man group are retarded by the column.[9] As indicated in Table I, all the Con A^- fractions were separated into the unbound fraction (DSA^-), the retarded fraction (DSA^r), and the bound fraction (DSA^+). It should be noted that the contents of DSA^+ fractions are higher in the order of metastasized carcinomas, primary lesions, and normal mammary gland; those of MC-1N, MC-2N, MC-1, and MC-2 are 2.0, 2.5, 1.4, and 1.3 times higher than that of NG-1, respectively. The contents of the DSA^r fractions were slightly higher in the metastasized carcinomas than others. Since there was no significant difference in the amounts of DSA^- fractions among the samples, the DSA^+ and DSA^r fractions were analyzed in detail after separation by Bio-Gel P-4 column chromatography (Fig. 2). Each of the DSA^+ fractions of NG-1 (solid line), MC-1 (dotted line), and MC-1N (dot-dashed line) was separated into several components named AN1 to AN3 (Fig. 2A), while each of the DSA^r fractions was eluted as a single peak named AN4 (Fig. 2B). It is evident that the contents of components AN2, AN3, and AN4 are higher in the order of MC-1N, MC-1, and NG-1. There was no significant difference in AN1. The DSA^+ fractions (Fig. 2C) and the DSA^r fractions (Fig. 2D) of MC-2 (dotted line) and MC-2N (dot-dashed line) were also separated in a similar manner, and the contents of AN2, AN3, and AN4 of MC-2N were also higher than those of MC-2, while the same amounts of AN1 were included in the two samples.

Structural analysis of components AN1 to AN4 Structures of the components AN1 to AN4 were studied by exoglycosidase digestion. Since the data for the five samples were quite similar, only the results of MC-1N will be described below.

As shown in Fig. 3A, sequential digestion with diplococcal β-galactosidase (solid line) specific for the Galβ1→4GlcNAc linkage,[18] diplococcal β-*N*-acetylhexosaminidase (dotted line) and jack bean β-*N*-acetylhexosaminidase (dot-dashed line), converted AN1 to fucosylated and non-fucosylated trimannosyl cores (Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ and Man_3·GlcNAcGlcNAc$_{OT}$, respectively), with release of three galactose, one *N*-acetylglucosamine,

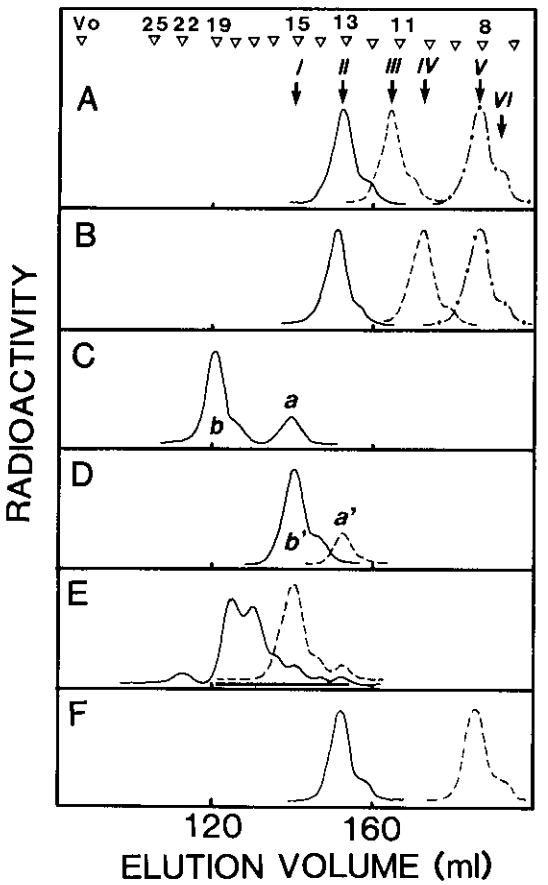

Fig. 3. Sequential glycosidase digestion of fractions AN1 to AN4. A and B, AN1 in Fig. 2A and AN4 in Fig. 2B sequentially incubated with diplococcal β-galactosidase (solid line), diplococcal β-*N*-acetylhexosaminidase (dotted line) and jack bean β-*N*-acetylhexosaminidase (dot-dashed line), respectively; C, AN2 in Fig. 2A incubated with endo-β-galactosidase from *F. keratolyticus*; D, peak a (dotted line) and peak b (solid line) in C incubated with diplococcal β-galactosidase; E, AN3 in Fig. 2A incubated with endo-β-galactosidase from *F. keratolyticus* (solid line) and the solid line peaks indicated by a bar in E digested with diplococcal β-galactosidase (dotted line); F, peak b′ in D incubated with diplococcal (solid line) and jack bean β-*N*-acetylhexosaminidase (dotted line). White triangles are the same as in Fig. 2 and arrows I to VI are the elution positions of authentic oligosaccharides; I, $GlcNAc_4{\cdot}Man_3{\cdot}GlcNAc{\cdot}Fuc{\cdot}GlcNAc_{OT}$; II, $GlcNAc_3{\cdot}Man_3{\cdot}GlcNAc{\cdot}Fuc{\cdot}GlcNAc_{OT}$; III, GlcNAc$\beta$1→6(GlcNAc$\beta$1→2)Man$\alpha$1→6(Man$\alpha$1→3)Man$\beta$1 → 4GlcNAc$\beta$1 → 4 (Fuc$\alpha$1 → 6) $GlcNAc_{OT}$; IV, Manα1→6(GlcNAcβ1→4Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$; V, $Man_3{\cdot}GlcNAc{\cdot}Fuc{\cdot}GlcNAc_{OT}$; VI, $Man_3{\cdot}GlcNAc{\cdot}GlcNAc_{OT}$.

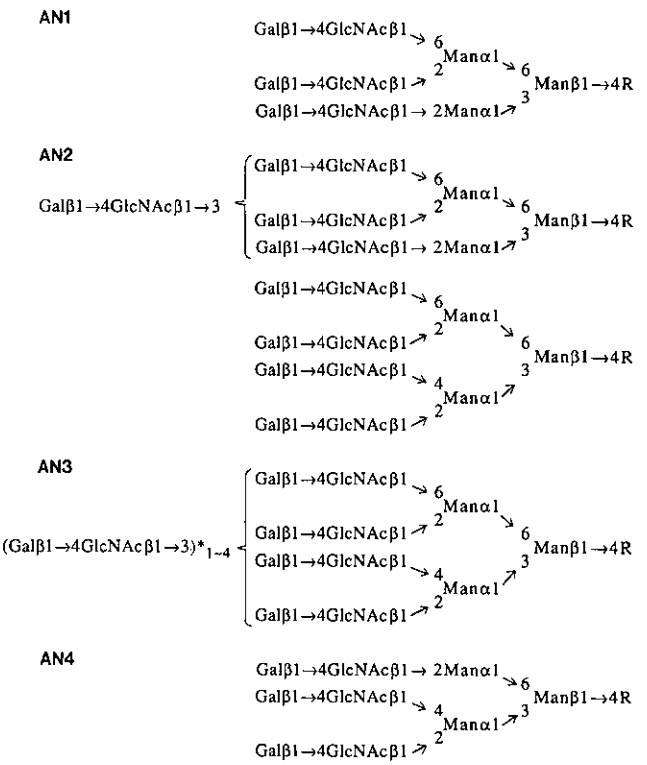

Fig. 4. Proposed structures of AN1 to AN4 in Fig. 2. R represents GlcNAcβ1→4($\pm$Fucα1→6)$GlcNAc_{OT}$. Some of the repeating units also occur as Galβ1→(4GlcNAcβ1→3Galβ1→)$_n$4GlcNAcβ1→3.

and two *N*-acetylglucosamine residues, respectively. By the same sequential glycosidase digestion, AN4 was also converted to the trimannosyl cores with release of three galactose (solid line), two *N*-acetylglucosamine (dotted line), and one *N*-acetylglucosaone residue (dot-dashed line), respectively (Fig. 3B). Considering these data with the substrate specificity of diplococcal β-*N*-acetylhexosaminidase, which cleaves the GlcNAcβ1→2Man linkage at a non-branched point or in the GlcNAcβ1→4GlcNAcβ1→2)Man group but not in the GlcNAcβ1→6(GlcNAcβ1→2)Man group,[19] it is evident that AN1 and AN4 are 2,6-branched and 2,4-branched triantennary complex-type sugar chains, respectively (Fig. 4).

By incubation with endo-β-galactosidase from *Flavobacterium keratolyticus*, which cleaves the internal β-galactosyl linkages of the *N*-acetyllactosamine repeating units such as Galβ1→ (4GlcNAcβ1→3Galβ1→)$_n$4-GlcNAc,[20] one-fourth of AN2 was converted to a component a with release of a Gal.GlcNAc.Gal group (Fig. 3C). Subsequent digestion with diplococcal β-galactosidase released two galactose residues (Fig. 3D, dotted line), and the resulting peak a′ was shown to be the same as the solid line peak in Fig. 3A in the same manner as described for AN1. Therefore, the endo-β-galactosidase-

sensitive component in AN2 is proposed to be 2,6-branched triantennary oligosaccharides containing *N*-acetyllactosamine repeating structure as shown in Fig. 4. On the other hand, the endo-β-galactosidase-resistant component b (Fig. 3C) was converted to component b′ with release of four galactose residues by diplococcal β-galactosidase digestion (Fig. 3D), and then to trimannosyl cores by sequential digestion with diplococcal (Fig. 3F, solid line) and jack bean β-*N*-acetylhexosaminidase (Fig. 3F, dotted line) releasing one and three *N*-acetylglucosamine residues, respectively. These results suggest that another component in AN2 is the non-repeated tetraantennary sugar chains (Fig. 4).

AN3 is composed of high-molecular-weight oligosaccharides with effective sizes larger than 22 glucose units (Fig. 2A). The size difference of each peak was approximately 3 glucose units, suggesting the presence of a series of oligosaccharides containing *N*-acetyllactosamine repeating units. By incubation with endo-β-galactosidase from *F. keratolyticus*, AN3 was converted to a mixture of smaller components as indicated by the solid line in Fig. 3E. Diplococcal β-galactosidase digestion of these components gave major radioactive products with the same elution position as the peak b′ in Fig. 3D (Fig. 3E, dotted line). The products at this stage were identified as degalactosylated tetraantennary sugar chains in the same manner (Fig. 3F) as described for the radioactive peak b′. The data indicate that AN3 is a mixture of tetraantennary sugar chains with different numbers of *N*-acetyllactosamine repeating units. When AN3 was digested with endo-β-galactosidase and the released oligosaccharides were labeled with NaB^3H_4, two smaller radioactive products corresponding to Gal·GlcNAc·Gal_{OT} and GlcNAc·Gal_{OT} were obtained in a ratio of 7:3 (data not shown). Therefore, the repeating units in AN3 have linear structures. The detection of GlcNAc·Gal_{OT} indicates the presence of long repeating units, Galβ1→(4GlcNAcβ1→3Galβ1→)$_n$4GlcNAc ($n \geq 2$), in some outer chains.

On the basis of these results described above and methylation analysis (data not shown), the structures of AN1 to AN4 are proposed to be as shown in Fig. 4. Thus, it is evident that tetra- and 2,6-branched triantennary oligosaccharides with *N*-acetyllactosamine repeating units, and non-repeated tetraantennary oligosaccharides (AN2 and AN3) are highly expressed in the order of metastasized carcinomas > primary carcinomas > normal mammary gland.

Stainings of membrane glycoproteins and tissues with DSA Membrane glycoproteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose paper and stained with DSA. As shown in Fig. 5, both MC-1N (lane 2) and MC-2N (lane 4) metastasized to lymph node contained several DSA-reactive glycoproteins including three major molecules with apparent molecular weights of 90,000 (GP90), 160,000 (GP160), and 210,000 (GP210). Primary carcinomas MC-1 (lane 1) and MC-2 (lane 3) showed weak reactivities compared with metastasized carcinomas. In contrast, DSA-reactive molecule was not detected in normal mammary gland under the conditions used here (data not shown). Thus, it is suggested that at least three glycoproteins, GP90, GP160, and GP210, are involved in altered glycosylation associated with progression of mammary carcinoma.

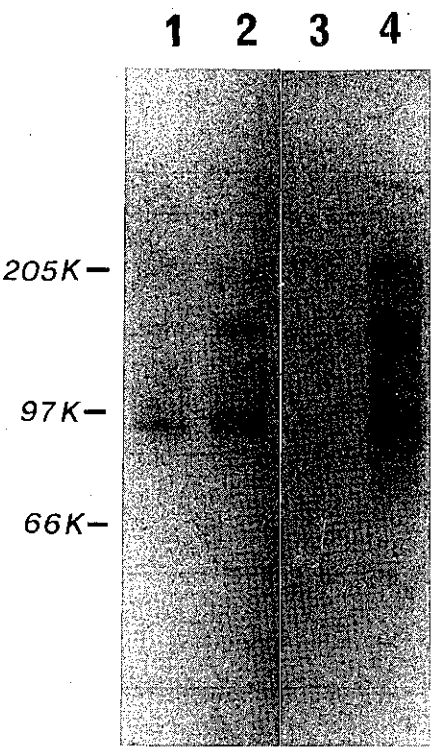

Fig. 5. DSA lectin staining of glycoproteins of mammary carcinoma tissues separated by SDS-polyacrylamide gel electrophoresis. Lane 1, MC-1 (primary); lane 2, MC-1N (metastasized); lane 3, MC-2 (primary); lane 4, MC-2N (metastasized). NG-1 did not show any detectable band. Numbers at the left-hand side indicate molecular weights estimated by using standard proteins.

Microscopic observation of specimens stained by the ABC method using biotinylated DSA revealed differences in staining pattern between normal mammary gland and mammary carcinomas (Fig. 6). Normal mammary gland showed almost no reactivity to DSA (Fig. 6A), or epithelial cells facing the luminal space of normal gland adjacent to carcinoma tissues of the primary lesion showed a faint luminal staining (Fig. 6B, short arrow). In contrast, carcinoma cells in the primary lesion (Fig. 6 B, long arrow and Fig. 6C) and those metastasized to the regional lymph node (Fig. 6D) exhibited a strong apical

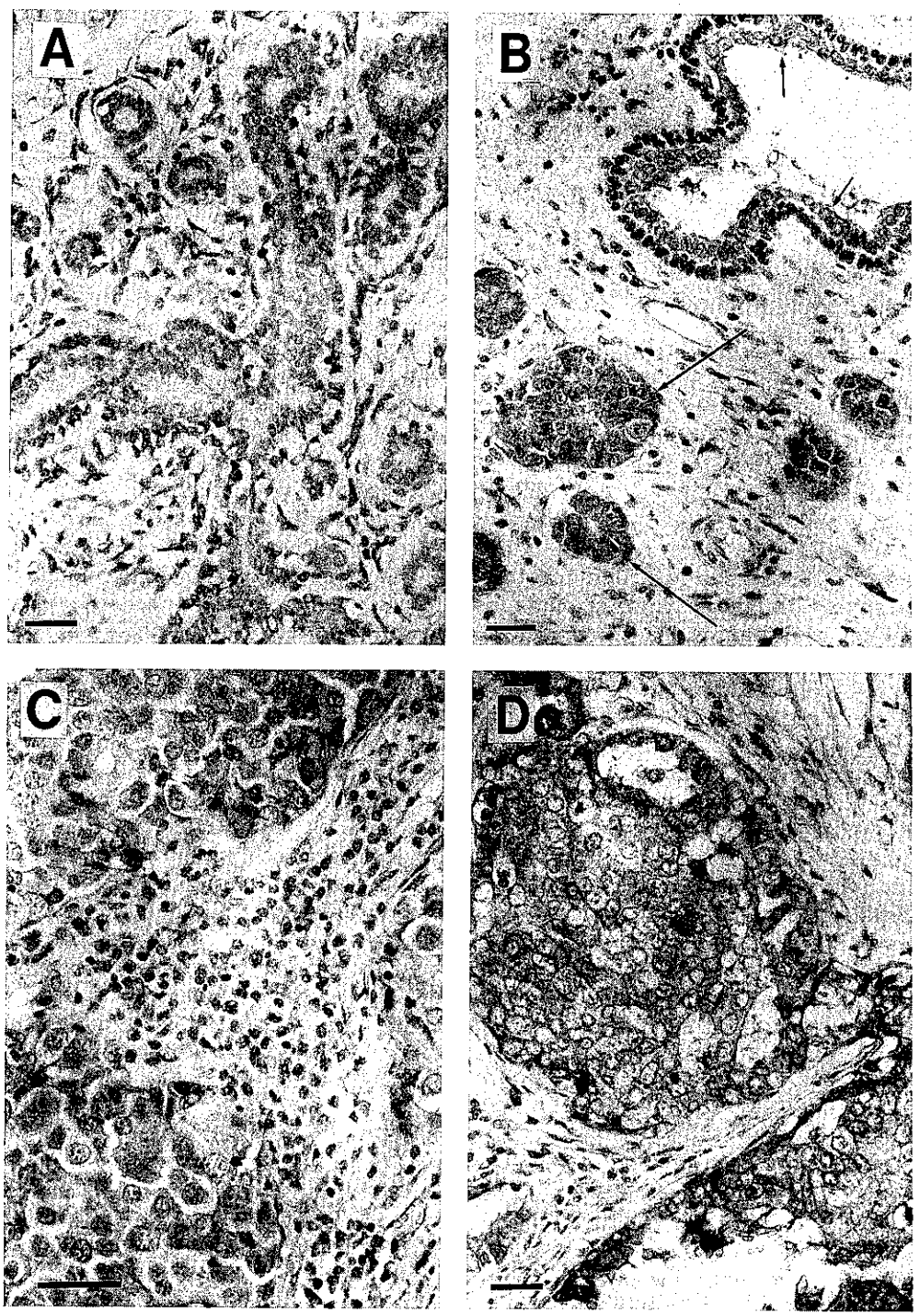

Fig. 6. Histochemical staining of mammary tissues by DSA. Tissues were stained with biotinylated DSA using the ABC method, counterstained with hematoxylin-eosin and observed under the microscope (×350, scale bar =50 μm). A, Normal mammary gland showing almost no reactivity. B, Invasive ductal carcinoma cells (long arrows) in primary lesion showing apical and cytoplasmic staining. Note a weak luminal staining of normal epithelial cells (short arrows). C, Invasive ductal carcinoma predominant with intraductal components in primary lesion showing apical and cytoplasmic staining. D, Invasive ductal carcinoma metastasized to regional lymph node showing apical and cytoplasmic staining.

staining and a diffuse intracytoplasmic staining. Interestingly, the borders between carcinoma cells seem to be stained. Thus, the results indicate that increased reactivity to DSA and difference in the cellular distribution of DSA-reactive glycoproteins develop in mammary carcinoma cells.

DISCUSSION

Cancer cells exhibit abnormal social behavior, such as uncontrolled cell growth, invasion and metastasis. Since these characteristics are considered to result from altered functions of cell membranes, extensive studies on cancer-associated glycosylation changes of membrane glycoproteins have been conducted. However, evidence generally has been obtained using rodent systems and cultured cells. In this study, we analyzed membrane glycoproteins obtained from normal human mammary gland, primary mammary carcinomas and their lymph node metastases. Fractionation of *N*-linked oligosaccharides released by hydrazinolysis using a DSA-Sepharose column revealed that primary carcinomas contain increased amounts of oligosaccharides which bind to this column as compared with normal mammary gland. Interestingly, the contents of DSA-bound oligosaccharides in metastasized carcinomas were higher than those in the primary carcinomas. Detailed structural analysis of these oligosaccharides indicated that the increase of the DSA-bound fraction is caused by higher expression of tetraantennary oligosaccharides with and without *N*-acetyllactosamine repeating units and 2,6-branched triantennary oligosaccharides with *N*-acetyllactosamine repeating units. Thus, the enhanced expression of the GlcNAcβ1→6 Manα1 → 6 and the Galβ1 → (4GlcNAcβ1 →3 Galβ1 →)$_n$4GlcNAc groups seems to be correlated with the progression of human mammary carcinoma. A recent study[7] showed that human mammary carcinomas express an elevated activity of *N*-acetylglucosaminyltransferase V responsible for formation of the GlcNAcβ1→6Manα1→6 group, as found in the rodent cell system.[21] Since formation of the *N*-acetyllactosamine repeating units initiated by the addition of GlcNAcβ1 →3 residue preferentially occurs on the Galβ1→4GlcNAcβ1 → 6(Galβ-1→4GlcNAcβ1→2)Manα1→6 group,[22] it is reasonable that both formation of the GlcNAcβ1→6 branching and elongation of the outer chains increased in mammary carcinomas. Our previous study indicated that a similar glycosylation change occurs in human esophageal squamous carcinoma.[8] It is suggested, therefore, that expression of the GlcNAcβ1→6 branching and elongated outer chains of *N*-linked complex-type sugar chains is regulated in association with malignant transformation of various types of human cells, as found in many rodent tumor cell systems.[1, 2, 5]

Histochemical analysis using DSA revealed different staining patterns between normal mammary gland and carcinoma tissues. In contrast to normal gland, showing a weak luminal surface staining, carcinomas showed an intense staining in the cytoplasm as well as at the cell surface. This difference will serve as an alternative indicator for diagnosis of mammary carcinoma. However, no significant difference in staining pattern was detected between primary and metastasized carcinomas. In this study, primary carcinomas were obtained from patients who had axillary lymph node metastasis. It is possible that these carcinoma cells in the primary lesion have already acquired some metastatic phenotypes. To test this possibility, it will be necessary to examine primary lesions of early mammary carcinoma. Accumulation of data on carcinomas from patients at different clinical stages as well as on benign mammary diseases will also be important for application of this glycosylation change to prognostic evaluation of the development and metastasis of mammary carcinoma.

Difference in cellular distribution of glycoproteins between human breast epithelia and mammary carcinomas has also been shown using other lectins with different sugar-binding specificities from that of DSA.[23, 24] In order to elucidate the presently unknown basis of these disorganized distributions of various cellular glycoproteins, it is important to examine what molecules are responsible for this phenomenon. The present study using lectin blot analysis indicated that three glycoproteins, GP-90, GP160, and GP210, are major carriers of DSA-reactive sugar chains. Our recent study using immunostaining suggests that GP-160 may correspond to carcinoembryonic antigen (CEA) (M. Nose *et al.*, unpublished data). Interestingly, it has also been shown that CEA obtained from liver metastasis of mammary carcinoma contains sialylated tetraantennary sugar chains as major components.[25] Quite recently, a CEA family including CEA and non-specific cross-reacting antigen (NCA) has been shown to mediate the Ca^{2+}-independent intercellular adhesion.[26–28] It is of interest that a large amount of CEA is located on adjacent cell membranes of colon carcinoma cells as well as on the membranes facing the lumen in both embryonic intestine and colon carcinoma, while a small amount of CEA is localized mostly in epithelial cell membranes facing the lumen in normal adult intestine.[26] This is notable in view of the present histochemical data obtained with DSA that only a faint luminal staining is found in normal mammary gland, while an intense staining is observed at the apical cytoplasmic surface including the borders between carcinoma cells in mammary carcinoma tissues. Whether CEA contributes to the altered cellular distribution of DSA-reactive glycoproteins associated with mammary carcinoma remains to be elucidated.

In addition, a diffuse intracytoplasmic staining of carcinoma cells with DSA is also of interest in relation to the recent finding that human lysosomal membrane-associated glycoproteins (LAMPs) are major carriers for polylactosamine glycans in many human cells.[29] It has been reported that the increased staining of gp130 with L-PHA, which is similar to DSA in sugar-binding specificity,[7] is associated with tumor progression in a rodent cell system,[5] and that this glycoprotein purified from mouse metastatic lymphoid tumor cell line called MDAY-D2 is a mouse counterpart of LAMP-1.[30] Therefore, it is possible to consider that the strong cytoplasmic staining of mammary carcinomas observed in this study results from accumulation of LAMPs in lysosomes. GP-90 is considered as a candidate for a LAMP. Detailed analysis of the DSA-reactive glycoproteins, the expression of which seems to be closely associated with the outbreak and possibly also the progression of human mammary carcinoma, is required as a next step.

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